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**Method of preventing wheat from mycotoxin contamination**

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(54) Title: METHOD OF PREVENTING WHEAT FROM MYCOTOXIN CONTAMINATION

(54) 発明の名称: 麦類のマイトキシン汚染抑制方法

(57) Abstract: It is intended to search for a cultivation method for reducing the content of mycotoxins in wheat which bring serious concerns for the qualities of wheat in the production sites and consumers' health risks. Namely, a method of reducing the content of mycotoxins in wheat characterized by comprising applying to wheat one or more compounds A selected from the group consisting of ammonium salts, primary to quaternary ammonium salts, alkali metal salts, alkaline earth metal salts and polyvalent metal salts of phosphorous acid and phosphorous acid esters.

(57) 要約: 小麦の生産現場における品質および消費者に対する健康被害リスク上、重要懸念事項となっている小麦中のマイトキシン汚染量を抑制する栽培方法を探索する。本発明は、亜リン酸及び亜リン酸エステル、アンモニウム塩、第 1～4 級アンモニウム塩、アルカリ金属塩、アルカリ土類金属塩及び多価金属塩よりなる群から選択される 1 種又は 2 種以上の化合物 A を麦類に施用することを特徴とする、麦類中のマイトキシン汚染量を抑制する方法を開示する。

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## Specification

Method for controlling mycotoxin contamination of cereals

### 5 Technical Field

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The present invention relates to a treatment method which intends to reduce contamination amount of mycotoxin produced by plant pathogenic fungi of cereals (hereinafter represented as "DON") in the cereals by using a composition  
10 for agri-horticulture containing one or more compounds A selected from the group consisting of ammonium salts, primary to quaternary ammonium salts, alkali metal salts, alkaline earth metal salts and polyvalent metal salts of phosphorous acid and phosphite ester as an active  
15 ingredient(s).

### Background Art

Fusarium head blight in cereals frequently occurs in cases of continuous cloudy weather and light rain, and  
20 furthermore high temperature from the heading date to the milk-ripe stage (Yukio Ozeki, Hiroshi Sasaki, Yoichi Amano, Hokkaido no Hatasaku Gijutu (Farming Technology in Hokkaido) -Cereals version-, published by Society for Agricultural Technique Propagation, page 209, 1978), and it  
25 is a plant disease which causes great damage to cereals in terms of yields, quality and so forth, and can not be avoided in this country due to the high amount of rainfall during the ripening period. As major pathogenic fungi thereof, *Fusarium graminearum*, *Fusarium culmorum*, *F.*  
30 *avenaceum* and *Microdochium nivale* have been specified. Although there is difference in occupancy depending on climate conditions and areas, complex infection is often observed in farm fields where the disease has occurred (Kunihei Kishi, Nippon Shokubutsu Byogai Daijiten  
35 (Comprehensive Dictionary of Plant Disease in Japan), published by Zenkoku Noson Kyoiku Kyokai (Association for

National Farming Village Education), page 74, 1998).

The plant pathogenic fungi which cause this disease produce more than one toxic metabolites referred to as mycotoxin, which contaminates crops during cultivating and poses a risk of ingestion by human and domestic animals through migration to harvestries and processed foods. Researches on mycotoxin in areas such as Europe, North America and East Asia where cultivation of the cereals is active have long history, DON has been specified as a toxic agent whose effects on human and animals are most concerned in terms of both toxicity and the contamination amount, and noticed worldwide. Ingestion of foods contaminated with DON causes acute poisoning where digestive organ symptoms including emesis and diarrhea are major symptoms. In Europe and North America, self-regulating values of DON contamination amount in grains have been established, and the system for intensified surveillance has been organized. While internationally rising momentum in DON surveillance, in 2002 in this country, the Ministry of Health, Labor and Welfare also established an interim standard value of 1.1 ppm for the DON contamination amount in the wheat, and announced that safety of the wheat distributed in the market was assured (Shokuhatu No. 0521001). In relation to the announcement, as an instruction notice for feed safety, the Ministry of Agriculture, Forestry and Fisheries established the interim acceptable value 4.0 ppm for deoxynivalenol in feeds supplied for cattle aged 3 months or more and 1.0 ppm that for domestic animals other than the above (press release). Before DON has been noticed as a fungus producing toxin, people have been protected from healthy risk due to fungus toxin by a law regulation that the contamination ratio of the mixed grains affected with Fusarium head blight shall be made less than 1% by visual check at the shipping stage of crude wheat. Thus, in the production fields of the cereals, the damages with Fusarium head blight have been reduced and inhibited by giving

fungicidal agents effective for pathogenic fungi of Fusarium head blight.

Chemicals effective for the pathogenic fungi of Fusarium head blight of the cereals commonly used at present are classified into several groups by their action mechanisms and chemical structures of active ingredients (Agricultural Chemical Handbook, 2001 version, published by Nippon Shokubutsu Boeki Kyokai). The SBI agents characterized by inhibition of biosynthesis of sterol universally present as a component of biological membrane of fungi include (RS)-1-p-chlorophenyl-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol (common name: tebuconazole), (1RS, 5RS)-5-(4-chlorobenzyl)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl) cyclopentanol (common name: metconazole), and 1-[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-ylmethyl]-1H-1,2,4-triazole (common name: propiconazole), and they are characterized by having a triazole skeleton in the chemical structures. They have been widely used since they are effective at low doses, rapidly permeate in plant bodies, are resistant to rain and low toxic for flower-visiting insects, and they have a high protection effect on the pathogenic fungi of Fusarium head blight of the cereals. Methyl=(E)-2-{2-[6-(2-cyanophenoxy)pyridine-4-yloxy]phenyl}-3-methoxyacrylate (common name: azoxystrobin) and methyl=(E)-2-methoxyimino[ $\alpha$ -(o-tolyloxy)-o-tolyl]acetate (common name: kresoxim-methyl) which were developed as strobilurine derivatives which are anti-mycotic antibiotics and structurally characterised by methoxyacrylate ester are classified into methoxyacrylate type fungicidal agents. The former exerts the protection effect by the inhibition of respiratory activity of the fungi, and the latter does it by the inhibition of cytochrome electron transfer system in mitochondria. The other synthetic fungicidal agents include 1,1'-iminodi(octamethylene)diguanidium=triacetate (common name: iminoctadine acetate). It is considered as

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the action mechanism that it causes destruction of a membrane lipid bilayer structure of fungi due to a surfactant-like feature derived from its salt structure. Furthermore, the relevance between the pathogenic fungi and drug efficacy has been researched, and it has been demonstrated that the triazole agents are effective for the 3 species of *F. graminearum*, *F. culmorum* and *F. avenaceum*, and that the methoxyacrylate agents are effective for *M. nivale*. As mentioned above, occurrence of the disease often accompanies mixture of the multiple pathogenic fungi, and thus, the occurrence of the disease has been protected and inhibited by performing rotation spray with taking advantage of characters of respective agents.

Along with attention for DON, as analysis of its contamination concentration has progressed, it has been being demonstrated that there is no direct relevance between an illness degree of the Fusarium head blight and the contamination amount of DON (Bai, G. H., Plattner R and Desjardins A., Relationship between visual scab rating and deoxynivalenol in wheat cultivars, The 1988 National Fusarium Head Blight Forum, Chapter 2, pages 21-25). It has been also demonstrated in recent years that *F. graminearum* and *F. culmorum* have DON production capacity but *F. avenaceum* and *M. nivale* do not produce DON. It has been reported that when *M. nivale* is controlled using the methoxyacrylate agent, *F. graminearum* and *F. culmorum* which are antagonized are increased resulting in facilitating the increase of DON contamination amount. Thus, the disease protection only by giving the fungicidal agents can not sufficiently prevent the DON contamination. Furthermore, in the production fields of the cereals, even when the Fusarium head blight of the cereals is controlled by combined application of various fungicidal agents, DON is frequently detected at more than 1.1 ppm. This troubles the producers. That is, the DON contamination amount can not be sufficiently reduced only by controlling the

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pathogenic fungi having a mycotoxin production capacity,  
which is a conventional technology. Also with respect to  
fosetyl described in the present statement, the fungicidal  
effect on the plant pathogenic fungi has been known  
5 publicly (US Patent No. 4,139,616, 1979; and JP 62-87504 A),  
but there is no mention at all for effects on plants  
contaminated with mycotoxin. Under such a circumstance, it  
is an actual state that establishment of a method of  
substantially reducing the DON contamination amount has  
10 been strongly desired in the production fields of the  
cereals.

The present inventors studied on compositions for  
agriculture which reduces contamination of wheat with  
mycotoxin, particularly a DON contamination amount to 1.1  
15 ppm or less or a low contamination amount as possible. As  
a result, a composition for agri-horticulture developed  
using one or more compounds A selected from the group  
consisting of ammonium salts, primary to quaternary  
ammonium salts, alkali metal salts, alkaline earth metal  
20 salts and polyvalent metal salts of phosphorous acid and  
phosphite ester as an active ingredient(s) has a low  
controlling effect on pathogenic fungi of Fusarium head  
blight of the cereals but has an excellent contamination  
inhibitory effect on a mycotoxin contamination,  
25 particularly the DON contamination, and have completed the  
invention.

Also, the inventors have shown a reduction of the DON  
contamination amount equal to or less than the standard  
value in mixed use with a fungicidal agent where DON  
30 contamination at high concentration of more than the  
standard value 1.1 ppm has been observed in the mixed use  
with fungicidal agent for agri-horticulture, and a further  
inhibitory effect on the DON contamination amount in the  
mixed use with a fungicidal agent which originally shows a  
35 low contamination level, have found an effect where the DON  
contamination amount is further reduced compared to a

single treatment of a fungicidal agent for agri-horticulture, and have completed the invention.

#### Disclosure of the Invention

5       The present invention is a method of reducing the contamination amount of mycotoxin (particularly deoxynivalenol) in cereals characterized in that one or more compounds A selected from the group consisting of ammonium salts, primary to quaternary ammonium salts, 10 alkali metal salts, alkaline earth metal salts and polyvalent metal salts of phosphorous acid and phosphate ester are given to the cereals.

Also, the invention relates to a method of reducing a contamination amount of mycotoxin (particularly 15 deoxynivalenol) in cereals characterized in that a combination of the above compound A is given to the cereals in combination with (suitably as a composition containing both as active ingredients) one or more fungicidal active components for agri-horticulture 20 (hereinafter referred to as the compound B).

Definitions of the specific embodiments of the invention as claimed herein follow.

According to a first embodiment of the invention, there is provided a composition containing one or more 25 compounds A selected from the group consisting of ammonium salts, primary to quaternary ammonium salts, alkali metal salts, alkaline earth metal salts and polyvalent metal salts of phosphorous acid and phosphite ester as an active ingredient(s), when used for 30 inhibiting mycotoxin contamination in cereals.

According to a second embodiment of the invention, there is provided a composition containing a compound A according to the first embodiment and one or more fungicidal active ingredient(s) for agri-horticulture.



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According to a third embodiment of the invention, there is provided a method for inhibiting mycotoxin contamination in cereals, the method comprising the step of administering to said cereals a composition containing one or more compounds A selected from the group consisting of ammonium salts, primary to quaternary ammonium salts, alkali metal salts, alkaline earth metal salts and polyvalent metal salts of phosphorous acid and phosphite ester as an active ingredient(s).

According to a fourth embodiment of the invention, there is provided use of the composition according to the first or second embodiments, in the manufacture of a formulation for use in the inhibition of mycotoxin contamination in cereals.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 represents test results of inhibitory effects on DON contamination by phosphite derivatives and alkyl phosphite derivatives (Example 1).

Fig. 2 represents test results of mixed use effects of potassium phosphite with various fungicides (Example 3).

#### Best Mode for Carrying Out the invention

Hereinafter, the invention is described in detail.

The ammonium salts, primary to quaternary ammonium salts, alkali metal salts, alkaline earth metal salts and polyvalent metal salts of phosphorous acid and phosphite ester, derived from phosphorous acid are not particularly limited so long as they reduce the contamination with mycotoxin, particularly DON, and for example, include

[text continues on page 7.]

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alkali metal salts and polyvalent metal salts of phosphorous acid and phosphite ester, and are suitably potassium phosphite and an aluminium salt (common name: fosetyl) of tris(ethylphosphonate).

5           Methods of assessing the inhibitory effect on mycotoxin contamination, particularly DON contamination in wheat of the invention can includes a method of determining or measuring by comparing a contamination concentration of DON, incidence rate of Fusarium head blight for panicles  
10           and incidence rate for spikelets in wheat, between a group where a single treatment with phosphorous acid and phosphite ester derivative was given, and a group where a treatment with mixed use of other fungicidal composition was given, and a control group without the treatment with  
15           drug, of the wheat cultivated in the field.

          It has been confirmed by such a method that the ammonium salts, primary to quaternary ammonium salts, alkali metal salts, alkaline earth metal salts and polyvalent metal salts of phosphorous acid and phosphite  
20           ester, derived from phosphorous acid have an excellent inhibitory effect on the mycotoxin contamination, particularly DON contamination, independently on controlling of the pathogenic fungi of the cereals.

          The compound B in the invention could be a usual  
25           fungicidal active compound for agri-horticulture, and is suitably the fungicidal active compound effective for wheat Fusarium head blight, such as a sterol biosynthesis inhibitor (SBI) having a triazole skeleton, azoxystrobin, kresoxim-methyl and iminoctadine.

30

#### EXAMPLES

          By citing below test examples using potassium phosphite and an aluminium salt (common name: fosetyl) of tris(ethylphosphonate) as the compounds A which are the  
35           active ingredients of the invention, the invention is more specifically described, but the invention is not limited

thereto.

Example 1: Single use ( $\pm$  conventional control) effect

Wheat (cultivar: Haruyutaka) was seeded on April 19, 2002, and cultivated according to a conventional  
5 cultivating standard (Hokkaido, Agriculture Department, 1995) to establish test sections with 6.75 m<sup>2</sup> per section (3 repeats). As the compounds A, potassium phosphite and an aluminium salt (common name: fosetyl) of tris(ethylphosphonate) were given, water solution of 0.038  
10 to 0.120 % in P<sub>2</sub>O<sub>5</sub> was prepared, and 100 L per 10 a was sprayed on leaves at next growth stage. That is, the first spray (June 28, flowering date), the second spray (July 8, 10 days after the flowering date, and the third spray (July 18, 20 days after the flowering date) were performed. A  
15 conventional control for the purpose of controlling plant pathogens and plant insects was performed together. That is, the first [June 20, azoxystrobin (2000 times dilution) + fenitrothion (1000 times dilution)], the second [tebuconazole (June 28, 2000 times dilution) + sumithione  
20 (1000 times dilution)], and the third [July 8, propiconazole (2000 times dilution) + fenitrothion (1000 times dilution)], the forth [July 17, tebuconazole (2000 times dilution) + fenitrothion (1000 times dilution)] were performed. As controls, the test section where only the  
25 conventional control was given and the test section without any control were established. Harvest was performed by harvesting the wheat in 4 m<sup>2</sup> in the test sections on August 9 (52 days after the heading date). After the harvest, the wheat was applied on a rice grader with 2.2 mm opening of  
30 screen to make selected grains, which were then pulverized by a rapid pulverizer to make whole grain powder . The whole grain powder was used as a sample for analysis. The DON contamination concentration was analyzed by ELISA method using commercially available Rida Screen Fast Don  
35 supplied from r-Biopharm.

Preparation of analysis sample solutions and analysis

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procedure are briefly described.

- To 5 g of the whole grain powder, 100 ml of water was added, and vigorously agitated for 10 min to make a DON extract solution.

5       - The DON extract solution was centrifuged at high speed, and the supernatant was used for the ELISA analysis.

- According to the method described on ELISA kit, various reagents were added, and subsequently an absorbance of each test solution was measured.

10       - The DON concentration of each test solution was read out from a standard curve made using DON standard solutions.

- The incidence rate of Fusarium head blight for panicles was calculated by counting the number of panicles  
15 in 1 m<sup>2</sup> of each section and the number of diseased panicles included therein.

The results of the present test are shown in Table 1.

Table 1. Inhibitory effect of phosphite derivative and alkyl phosphite derivative on DON contamination (2002, Haruyutaka)

|   | Treatment           |               |                      | DON concentration (ppm) | Incidence rate of Fusarium head blight for panicles(%) |
|---|---------------------|---------------|----------------------|-------------------------|--|
|   | Active ingredient A | Concentration | Control system       |                         |  |
| 1 | Potassium phosphite | 0.038%        | Conventional control | 2.41                    | 0.3  |
| 2 |                     | 0.070%        | Conventional control | 0.59                    | 0.0  |
| 3 |                     | 0.112%        | Conventional control | 0.39                    | 0.9  |
| 4 |                     |               | No control           | 0.69                    | 1.2  |
| 5 | Fosetyl             | 0.120%        | Conventional control | 0.84                    | 0.0  |
| 6 | Potassium phosphate | 0.112%        | Conventional control | 4.66                    | 0.0  |
| 7 |                     |               | Conventional control | 6.16                    | 0.9  |
| 8 |                     |               | No control           | 8.69                    | 4.9  |

Note) The concentration of the active ingredient A is the concentration converted into  $P_2O_5$ .

Note) The incidence rate of Fusarium head blight for panicles was calculated by counting the number of panicles in 1 m<sup>2</sup> of each section and the number of diseased panicles included therein.

10

DON was detected at highly contaminated levels which were much higher than the interim standard value, 1.1 ppm that the Ministry of Health, Labor and Welfare had presented from the section with no control and the section with conventional control alone using the fungicidal composition for controlling of Fusarium head blight of the cereals. On the other hand, inhibition of DON contamination depending on treated concentrations was observed in the sections treated with potassium phosphite and the aluminium salt (common name: fosetyl) of tris(ethylphosphonate), and their contamination levels were

20

lower than 1.1 ppm. It is worthy of special mention that even in the section without any control where the conventional control is not performed, the DON contamination concentration is sufficiently reduced by giving potassium phosphite. Furthermore, even when potassium phosphate which is a salt of normal phosphoric acid is given, the DON inhibitory effect is low, and thus it is obvious that the phosphite and alkyl phosphite derivatives play an important role. Even when the incidence rate of Fusarium head blight for panicles was at almost the same degree, DON at high concentration was detected in the section without treatment with potassium phosphite. This way, it is evident that the concentration of DON contamination is reduced by the treatment with phosphorous acid and alkyl phosphorous acid and the derivatives thereof.

Example 2: Toxin-inhibitory effect by seeds diseased with Fusarium head blight in Example 1

Healthy grains and grains diseased with Fusarium head blight were screened from the crude wheat obtained in Example 1, and the DON contamination concentration of the whole grain powder thereof was analyzed by the ELISA method in the same way as shown in Example 1.

The results of the present test are shown in Table 2.

Table 2. DON contamination concentration of grains diseased with Fusarium head blight

|   | Treatment content   |               |                      | DON concentration (ppm) |                |
|---|---------------------|---------------|----------------------|-------------------------|----------------|
|   | Active ingredient A | Concentration | Control system       | healthy grain           | diseased grain |
| 1 | Potassium phosphite | 0.038%        | Conventional control | 0.05                    | 76.6           |
| 2 |                     | 0.070%        | Conventional control | 0.04                    | 44.7           |
| 3 |                     | 0.112%        | Conventional control | 0.04                    | 25.4           |
| 4 |                     |               | No control           | 0.04                    | 75.6           |
| 5 | Fosetyl             | 0.120%        | Conventional control | 0.96                    | 11.0           |
| 6 | Potassium phosphate | 0.112%        | Conventional control | 0.06                    | 86.9           |
| 7 |                     |               | Conventional control | 1.72                    | 90.0           |
| 8 |                     |               | No control           | 0.73                    | 173.0          |

Note) The concentration of the active ingredient A is the concentration converted into  $P_2O_5$ .

5        Note) The grains colored with scarlet or orange inherent for Fusarium head blight and shrink grains were sorted as the diseased grain, and the grains other than them were sorted as the healthy grains.

10        The concentration of DON contamination of the healthy grains varied almost within lower range. On the other hand, in the diseased grains with Fusarium head blight, DON was detected at an extremely high concentration as expected. However, in the crude wheat determined to be the diseased

15        grains, difference was observed in the concentration of DON contamination. That is, in the section given the treatment with potassium phosphite, the DON contamination concentration was decreased depending on the given

20        concentration of potassium phosphite. This way, it is obvious that giving potassium phosphite reduces the DON contamination concentration in the wheat regardless the presence or absence or the degree of the disease with

Fusarium head blight of cereals, and further that the reduced degree of the DON contamination depends on the given concentration of potassium phosphite.

5 Example 3: Mixed use (1)

Wheat (cultivar: Haruyutaka) was seeded on April 23, 2002, and cultivated according to a conventional cultivating standard (Hokkaido, Agriculture Department, 1995) to establish test sections with 10 m<sup>2</sup> per section (3  
10 repeats). A suspension containing potassium phosphite and various fungicidal agents was prepared in a liquid mixture made up of a spreading agent (Gramine S: supplied from Hokkai Sankyo Co., Ltd.) and water. Using potassium  
phosphite as the compound A, 0.070% P<sub>2</sub>O<sub>5</sub>, and the 0.006 to  
15 0.025% fungicidal agent for agri-horticulture for the wheat as the compound B, water solution containing them was prepared, and 100 L thereof per 10 a was sprayed on leaves in the following growth stages. That is, the first spray  
(June 24, heading date), the second (June 30, flowering  
20 stage), the third (July 9) were performed. The harvest was performed on August 12 (50 days after the heading date) by harvesting the wheat in 3.4 m<sup>2</sup> in each section. After the harvest, the wheat was applied on a rice grader with 2.2 mm opening of screen to make selected grains, which were then  
25 pulverized by a rapid pulverizer to make whole grain powder. The whole grain powder was used as a sample for analysis. The DON contamination concentration was analyzed using HPLC-UV method in officially fixed methods shown by the Minister of Health, Labor and Welfare. Quantitative  
30 analysis was performed by repeating three times, and the quantitative value was an average value thereof.

Preparation of analysis sample solutions and the analysis procedure are briefly described.

- To 50 g of the whole grain powder, 85% acetonitrile  
35 was added, vigorously agitated for 30 min, and subsequently sonicated for 10 min.



- After eliminating impurities with filter paper, a filtrate was purified using a pretreatment column MultiSep #227 to make a solution for analysis.

5     - The test solution for analysis was injected into high performance liquid chromatography, and DON was detected by ultraviolet rays.

- The DON concentration of each test solution was read out from a standard curve made using DON standard solutions.

10     - The incidence rates of Fusarium head blight for panicles and spikelets were surveyed for 100 panicles in each section.

The results of the present test are shown in Table 3.

Table 3. Mixed use effects of potassium phosphite and various fungicidal agents

|    | Presence or absence of active ingredient A, potassium phosphite | Active ingredient B, Concentration of active ingredient | DON concentration (ppm) | Incidence rate for panicles (%) | Number of diseased spikelets/panicle |
|----|---|---|-------------------------|---------------------------------|--------------------------------------|
| 1  | ×   | Tebuconazole<br>0.020%                                  | 0.39                    | 0.3                             | 0.003                                |
| 2  | ○   |   | 0.38                    | 1.0                             | 0.010                                |
| 3  | ×   | Metconazole<br>0.006%                                   | 0.34                    | 0.7                             | 0.007                                |
| 4  | ○   |   | 0.10                    | 1.0                             | 0.010                                |
| 5  | ×   | Propiconazole<br>0.017%                                 | 1.01                    | 1.0                             | 0.010                                |
| 6  | ○   |   | 0.72                    | 2.7                             | 0.027                                |
| 7  | ×   | Azoxystrobin<br>0.010%                                  | 4.65                    | 2.3                             | 0.027                                |
| 8  | ○   |   | 0.96                    | 2.0                             | 0.023                                |
| 9  | ×   | Kresoxim-methyl<br>0.021%                               | 1.05                    | 0.3                             | 0.003                                |
| 10 | ○   |   | 0.51                    | 0.7                             | 0.007                                |
| 11 | ×   | Iminoctadine acetate<br>0.025%                          | 1.04                    | 1.7                             | 0.017                                |
| 12 | ○   |   | 0.20                    | 3.0                             | 0.030                                |
| 13 | ○   |   | 0.77                    | 4.3                             | 0.050                                |
| 14 | Non-treatment   |   | 3.69                    | 6.7                             | 0.138                                |

Note) 0.07% P<sub>2</sub>O<sub>5</sub> as potassium phosphite; circle: treated, crisscross: untreated

5 Note) The incidence rates for panicles and spikelet rates were surveyed for 100 panicles in each test section.

#### Example 4: Mixed use (2)

10 Wheat (cultivar: Haruyutaka) was seeded on May 4, 2003, and cultivated according to a conventional cultivating standard (Hokkaido, Agriculture Department, 1995) to establish test sections with 10 m<sup>2</sup> per section (3 repeats). A suspension containing potassium phosphite and

various fungicidal agents was prepared in a liquid mixture made up of a spreading agent (Gramine S: supplied from Hokkai Sankyo Co., Ltd.) and water. Using potassium phosphite as the compound A, 0.070%  $P_2O_5$ , and the 0.030 to 5 0.125% fungicidal agent for agri-horticulture for the wheat as the compound B, an water solution containing them was prepared, and 100 L thereof per 10 a was sprayed on leaves in the following growth stages. That is, the first spray (July 1, flowering stage), the second (July 7), the third 10 (July 14) were performed. Harvest was performed on August 25 by harvesting all wheat in each test section. After the harvest, using a sample divider, the wheat was equally divided, and was applied on a rice grader with 2.2 mm opening of screen to make selected grains, which were then 15 pulverized by a rapid pulverizer to make whole grain powder. The whole grain powder was used as a sample for analysis. The concentration of DON contamination was analyzed by the ELISA method in the same way as shown in Example 1.

Table 4. Mixed use effects of potassium phosphite and various fungicidal agents

|    | Presence or absence of active ingredient A, potassium phosphite | Active ingredient B, Concentration of active ingredient | DON concentration (ppm) | Incidence rate for panicles (%) | Number of diseased spikelets/ panicle |
|----|---|---|-------------------------|---------------------------------|---------------------------------------|
| 1  | ×   | Tebuconazole<br>0.020%                                  | 0.16                    | 7.0                             | 7.3                                   |
| 2  | ○   |   | 0.08                    | 5.0                             | 5.0                                   |
| 3  | ×   | Propiconazole<br>0.017%                                 | 0.57                    | 4.7                             | 5.0                                   |
| 4  | ○   |   | 0.11                    | 4.7                             | 5.0                                   |
| 5  | ×   | Azoxystrobin<br>0.010%                                  | 0.83                    | 4.3                             | 4.7                                   |
| 6  | ○   |   | 0.50                    | 3.7                             | 4.0                                   |
| 7  | ×   | Trifloxystrobin<br>0.025%                               | 0.35                    | 4.3                             | 5.0                                   |
| 8  | ○   |   | 0.12                    | 3.0                             | 3.0                                   |
| 9  | ×   | Iminoctadine<br>albesilate<br>0.030%                    | 0.22                    | 7.3                             | 8.0                                   |
| 10 | ○   |   | 0.00                    | 2.7                             | 2.7                                   |
| 11 | ×   | sulfur<br>0.125%  | 1.85                    | 8.7                             | 10.3                                  |
| 12 | ○   |   | 0.43                    | 8.7                             | 8.7                                   |
| 13 | ○   |   | 0.57                    | 10.3                            | 11.0                                  |
| 14 | Non-treatment   |   | 2.15                    | 20.4                            | 23.8                                  |

Note) Potassium phosphite is 0.070% in P<sub>2</sub>O<sub>5</sub>. ○:with treatment, ×:without treatment

5 Note) The incidence rates for panicles and spikelets were surveyed for 100 panicles in each test section .

10 In the same way as shown in Example 3, in the mixed use with any of the fungicidal agents for agri-horticulture, both the incidence rate for panicle and the incidence rate for spikelet exhibited high values compared to a single treatment, but the DON contamination amount was reduced.

Worthy of special mention is the result in section given the single agent treatment with the compound A, where the disease rate of Fusarium head blight was high, nonetheless, the DON contamination amount was at a low level.

5

Example 5: Effects on fungal growth and toxin production  
(1)

10 A water solution containing 5.600% potassium phosphite was prepared, soaked into gas-sterilized wheat seeds of "Hokushin" , and subsequently Fusarium head blight pathogenic fungus of the cereals, *Fusarium graminearum* having DON production capacity was inoculated, which were then cultured at 27°C. On days 7, 14, 21 and 28 in the culture, the amount of Fusarium head blight pathogenic  
15 fungus of the cereals and the amount of DON production present in wheat grains were analyzed.

The preparation of ergosterol analysis sample solutions and the analysis procedure are briefly described.

20 - To 5 g of the culture, 80 ml of ethanol was added, and pulverized using a rapid pulverizer.

- The ethanol solution containing the pulverized culture was vigorously agitated for 30 min to extract ergosterol.

25 - After eliminating impurities with filter paper, a filtrate was concentrated under reduced pressure, dried, solidified and dissolved again in 10 ml of ethanol to make an analysis sample solution.

30 - The analysis sample solution was injected into high performance liquid chromatography, and ergosterol was detected by ultraviolet rays.

The preparation of DON analysis sample solutions and the analysis procedure are briefly described.

- To 4 g of the culture, 80 ml of distilled water was added, and pulverized using a rapid pulverizer.

35 - The water solution containing the pulverized culture was vigorously agitated for 30 min to extract DON.

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- A part of the extract solution was centrifuged, and the supernatant was used for the ELISA analysis.

- According to the instruction manual in the ELISA kit, various reagents were added, and subsequently an absorbance of each test solution was measured.

- The DON concentration of each test solution was read out from standard curve made using DON standard solution.

Table 5. Change over time of fungal growth and DON production in wheat treated with potassium phosphite

| Treatment                   | Analysis item                    | Days of culturing |         |         |         |
|-----------------------------|----------------------------------|-------------------|---------|---------|---------|
|                             |                                  | 7 days            | 14 days | 21 days | 28 days |
| Potassium phosphite<br>5.6% | DON(ppm)                         | ND.               | ND.     | ND.     | ND.     |
|                             | Ergosterol<br>peak area<br>value | 17952             | 115921  | 372377  | 350972  |
| Non-<br>treatment           | DON(ppm)                         | 6.3               | 4.43    | 4.29    | 37.8    |
|                             | Ergosterol<br>peak area<br>value | 150453            | 271882  | 367236  | 559014  |

Note) ND means that the analytical value is less than the detection limit (0.222 ppm) of the ELISA analysis kit .

The amount of ergosterol which was an indicator of the amount of fungi was continuously increased during the culture in the non-treatment whereas reached a plateau on the 21st day in the treatment with potassium phosphite. With respect to the amount of DON production, in the non-treatment, the DON production was initiated at an early phase of the culture and remarkably increased on the 28th day. On the contrary, in the treatment with potassium phosphite, DON was not detected during the entire period of the culture. It is obvious that application of potassium phosphite has a high inhibitory effect on the DON production regardless of the presence or absence or the

proliferation degree of the fungi for the Fusarium head blight of cereals.

Example 6: Effects on fungal growth and toxin production

5 (2)

10 A water solution containing 0.056% to 2.800% potassium phosphite was prepared, soaked into gas-sterilized wheat seeds of "Hokushin", and subsequently Fusarium head blight pathogenic fungus of the cereals, *Fusarium graminearum* having DON production capacity was inoculated to the wheat seeds, which were then cultured at 27°C. On day 28 in the culture, the amount of Fusarium head blight pathogenic fungus of the cereals and the amount of DON production present in wheat grains were analyzed by  
15 the methods in the same way as shown in Example 5.

Table 6. Effects of potassium phosphite concentration on fungal amount of Fusarium head blight pathogenic fungus of the cereals and the amount of DON production

| Treatment concentration | DON (ppm) | Ergosterol peak area |
|-------------------------|-----------|----------------------|
| 0.056%                  | 1.60      | 1834861              |
| 0.112%                  | 0.41      | 1769582              |
| 0.280%                  | ND.       | 1810454              |
| 0.560%                  | ND.       | 1921693              |
| 2.800%                  | ND.       | 85891                |
| Non-treatment           | 46.1      | 3601748              |

20 Note) ND means that the analytical value is less than the detection limit (0.222 ppm) of the ELISA analysis kit.

25 Considering the amount of ergosterol as the indicator of the amount of fungi, in the treatment with the water solution of 0.056 to 0.560% potassium phosphite, the inhibitory effect on the fungal amount at a moderate degree

and similar degree as compared to that in the non-treatment (water only) was observed, and the remarkable inhibitory effect on the fungal amount was observed in the treatment with the water solution of 2.800% potassium phosphite. Concerning DON amount, in the range of concentrations from 0.056 to 2.800% of potassium phosphite in the water solutions, it was significantly lower than that in the non-treatment. It is obvious that the application of potassium phosphite has a high inhibitory effect on the DON production regardless of the presence or absence or the proliferation degree of the fungi for the Fusarium head blight of cereals.

#### Industrial Applicability

According to the invention, it has been demonstrated that an excellent inhibitory effect on mycotoxin contamination, particularly DON contamination can be had regardless of the presence or absence or the degree of the disease with Fusarium head blight of cereals although the prevention effect on Fusarium head blight pathogenic fungi of cereals is low by spraying one or more compounds A selected from the group consisting of ammonium salts, primary to quaternary ammonium salts, alkali metal salts, alkaline earth metal salts and polyvalent metal salts of phosphorous acid and phosphite ester onto the growing wheat. Also, it has been demonstrated that the contamination amount of mycotoxin is further reduced by the mixed use of the other fungicidal agent for agriculture compared to the use of the single fungicidal agent alone.

The term "comprise" and variants of the term such as "comprises" or "comprising" are used herein to denote the inclusion of a stated integer or stated integers but not to exclude any other integer or any other integers, unless in the context or usage an exclusive interpretation of the term is required.

Any reference to publications cited in this specification is not an admission that the disclosures constitute common general knowledge in Australia.



The claims defining the invention are as follows:

1. A composition containing one or more compounds A selected from the group consisting of ammonium salts, primary to quaternary ammonium salts, alkali metal salts, alkaline earth metal salts and polyvalent metal salts of phosphorous acid and phosphite ester as an active ingredient(s), when used for inhibiting mycotoxin contamination in cereals.

2. The composition according to claim 1 wherein the compound A is an alkali metal salt or a polyvalent metal salt of phosphorous acid or phosphite ester.

3. The composition according to claim 1 wherein the compound A is an aluminium salt of tris(ethylphosphonate).

4. The composition according to claim 1 wherein the compound A is potassium phosphite.

5. A composition containing a compound A according to any one of claims 1 to 4 and one or more fungicidal active ingredient(s) for agri-horticulture.

6. The composition according to claim 5, wherein the fungicidal active ingredient(s) for agri-horticulture is selected from the group consisting of an inhibitor of sterol biosynthesis having a triazole skeleton, a methoxyacrylate based fungicidal agent, a fungicidal agent which causes destruction of a membrane lipid bilayer structure of fungi and sulfur.

7. The composition according to claim 5, wherein the fungicidal active ingredient(s) for agri-horticulture is selected from the group consisting of tebuconazole, metconazole, propiconazole, azoxystrobin, kresoxim-methyl, iminoctadine acetate, iminoctadine albesilate, trifloxystrobin and sulfur.

8. The composition according to claim 5, wherein the

fungicidal active ingredient(s) for agri-horticulture is azoxystrobin, iminoctadine acetate or iminoctadine albesilate.

9. The composition according to any one of claims 1 to 8, wherein said mycotoxin is deoxynivalenol.

10. A method for inhibiting mycotoxin contamination in cereals, the method comprising the step of administering to said cereals a composition containing one or more compounds A selected from the group consisting of ammonium salts, primary to quaternary ammonium salts, alkali metal salts, alkaline earth metal salts and polyvalent metal salts of phosphorous acid and phosphite ester as an active ingredient(s).

11. Use of the composition according to any one of claims 1 to 9, in the manufacture of a formulation for use in the inhibition of mycotoxin contamination in cereals.

12. A composition as defined in claim 1 and substantially as hereinbefore described with reference to one or more of the accompanying examples.

13. A composition as defined in claim 5 and substantially as hereinbefore described with reference to one or more of the accompanying examples.

14. A method of inhibiting mycotoxin contamination in cereals, the method comprising the step of administering to said cereals a composition according to claim 12 or claim 13.

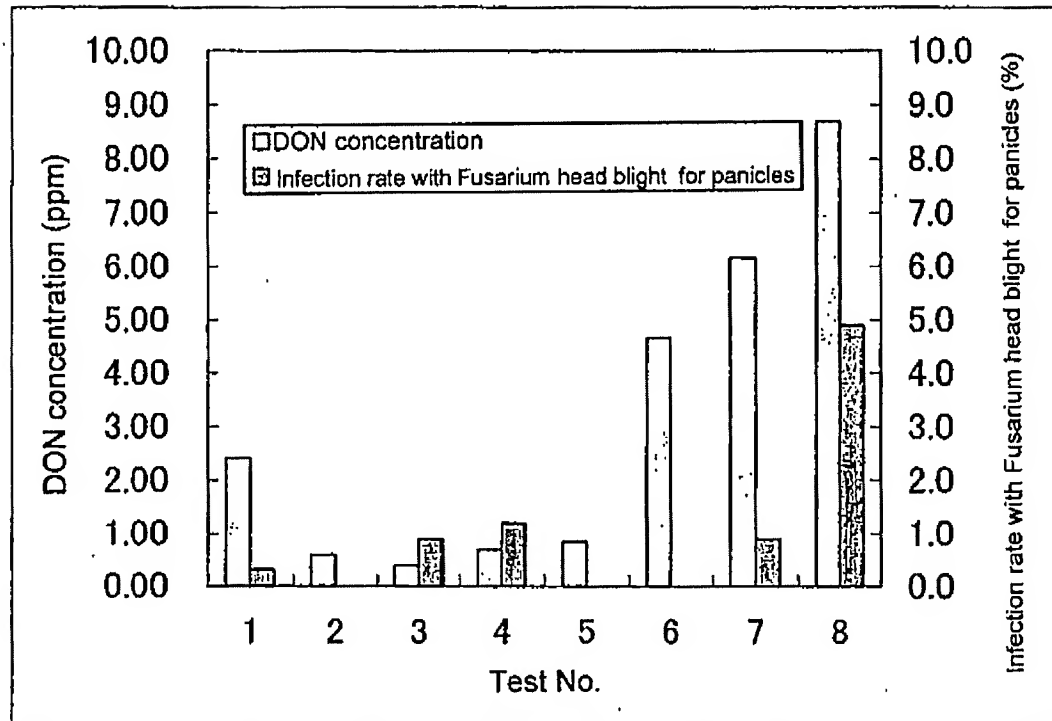
15. Use of a composition according to claim 12 or claim 13 in the manufacture of a formulation for use in the inhibition of mycotoxin contamination in cereals.

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Fig. 1



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Fig. 2

